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ISOTOPES IN NUTRITION RESEARCH

I. The Intermediate Metabolism of Fats, Proteins and Carbohydrates:

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ISOTOPES IN NUTRITION RESEARCH

I. The Intermediate Metabolism of Fats, Proteins and Carbohydrates:

The release of atomic energy represents the dramatic culmination of the early experiments of Becquerel and of the Curies whose discovery of radioactivity almost fifty years ago resulted in the birth of a new science–nuclear physics. The science which has given us the atomic bomb has also made available to medicine, biochemistry and nutrition research one of the greatest scientific tools since the invention of the microscope–*isotopic tracers.*

Isotopes are different forms of the same element with almost identical properties. They are physiologically indistinguishable to the living cell but can be readily detected because of their differences in mass (atomic weight) or by characteristic radiation.

The discovery and isolation of naturally occurring stable isotopes, and the creation of new, radioactive forms of the elements through nuclear bombardment, have made possible a unique method for labeling or tagging compounds and thereby for tracing their course through the animal body. By this means new light has been shed on the complex mechanisms involved in metabolism and nutrition and a dramatic new concept of the body economy has arisen–that of the *dynamic state of body constituents* (1).

Origin and Principles of the Tracer Technique:

The chemical reactions occurring in metabolism have been a matter of great interest to biochemists for many years. Early methods for studying the assimilation, distribution, conversion and excretion of food products were limited and inadequate. In some studies, abnormally large quantities of a metabolite were fed, so that a corresponding tissue increase in the element could be detected in the body. This procedure often resulted in a disturbance of normal equilibrium conditions, thereby minimizing the significance of the results obtained. A further drawback consisted of the inability of chemical methods to differentiate between the atoms or molecules derived from the ingested material and similar ones already present in the living tissue. In illustration of this point, Hamilton (2) cites the following example:

> "The increase in excretion of phosphorus . . . following administration of this element is made up in part by the phosphorus atoms already in the body, which were displaced by the administered element, and in part

by a portion of the phosphorus given to the animal. The classical procedures of chemistry and physics do not permit the investigator to determine what proportion of the phosphorus atoms in the excreta came from the administered material and the fraction that was displaced from the pre-existing phosphorus in the tissues. This limitation applies to all metabolic investigations in which the elements or compounds are constituents of the organism that is being studied."

Attempts to overcome this difficulty resulted in the feeding of tagged compounds whose presence could be readily detected and whose derivatives could not be confused with constituents derived from normal tissues. One method of tagging compounds, for example, was the replacement of one or more hydrogen atoms in a fatty acid molecule by the easily detectable halogen, bromine. This resulted in the production of a biologically unnatural compound. Here again the results were unsatisfactory because the highly selective tissue cells usually respond differently to such abnormal compounds.

A little more than two decades ago Hevesy (3) successfully employed the natural radioactive isotope of lead, radium D, for the study of the course of lead through plants. This use of an isotopic tracer represented the first successful method for labeling a biological compound.

The usefulness of isotopic tracers is based on the fact that isotopes are almost identical in physical and chemical properties and are, therefore, physiologically indistinguishable to the living cell. There are two types of isotopes, stable and radioactive. Stable isotopes are distinguished from one another only by a difference in mass or atomic weight*. They can be detected and estimated quantitatively by the mass spectrograph or by density measurements. Radioactive isotopes, on the other hand, in addition, undergo characteristic nuclear transformations with the emission of alpha, beta or gamma radiation. These can be detected and measured by the Geiger-Müller counter and similar devices.

Radioactive isotopes have the advantage of being rapidly and easily detectable even in very high dilution. Certain of the radioactive isotopes, however, may be limited in their usefulness as biological tracers either because of unavailability or because of the short half life** of the element. Thus, in tracer studies involving nitrogen metabolism, for example, the stable isotope, N^{15}, is preferred to

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*Thus, for example, deuterium, D, an isotope of hydrogen, has an atomic weight of 2 as compared to hydrogen, H, which has a mass of 1. (Hence the terms "heavy" hydrogen, D_2, and "heavy" water, D_2O).

**The half-life of a radioactive element is that time interval during which the element loses one-half of its radioactivity. Thus, radioactive nitrogen with a half-life of ten minutes would lose half its original activity in the first ten minutes, half of the remaining activity in the next ten minutes, and so on. The radioactive isotope of hydrogen, tritium, H^3, on the other hand, has a half-life of several years, but is not available in sufficient quantity for general use.

either of the radioactive forms, N^{16} and N^{13}, which have half-lives of eight seconds and ten minutes, respectively. A further precaution in the use of radioactive isotopes is the necessity for using a quantity sufficiently small so as to prevent physiological disturbance due to the radiations. Stable isotopes, on the other hand, are somewhat less sensitive to detection in high dilution, but the methods for their detection are more accurate. Also, stable isotopes do not deteriorate with the passage of time. This permits not only longer term experiments, but enables the chemist to prepare and keep on hand for use at any time such labeled compounds as may be used in his work.

Because of the difficulty of separating naturally occurring stable isotopes and because of the unavailability of radioactive forms of the more common elements, little progress was made using the tracer technique after Hevesy until 1932 when Urey (4) discovered and later made available deuterium, the naturally occurring stable isotope of hydrogen ("heavy" hydrogen). This was soon followed by the isolation of the stable isotopes of carbon, oxygen, nitrogen and sulfur.

With the availability of isotopic forms of these common elements there began a number of investigations on the fate of organic compounds in the animal body. These investigations received tremendous impetus with the discovery in 1934 by Curie and Joliot (5, 6) of artificially induced radioactivity. They found that certain elements, upon bombardment with particles emitted by naturally radioactive materials, such as radium, were converted to new radioactive isotopes of the various elements. This discovery made possible the creation of artificially radioactive isotopes of almost all of the elements. The work of the Joliot-Curies was greatly extended with the development by Lawrence and coworkers (7, 8) of the powerful atom-smashing machine, the cyclotron, which permits the large-scale production of radioactive isotopes. An important by-product of recent developments in nuclear fission is the creation of a new and large source of supply of radioactive isotopes.

Method of Using Isotopes: In metabolic studies using isotopes, the physiological substance to be investigated is synthesized so that one or more of its component atoms are replaced by an abnormally high concentration of the corresponding isotopic atoms. In this way the physiological test substance is "tagged" or "labeled" and its subsequent distribution in the various body tissues can then be followed. The tagged compound is fed. Other compounds later synthesized in the body are isolated. If these latter compounds show an excess above normal of the isotope fed, this is taken as an indication of conversion of the test compound fed to the one isolated. It is possible in this way

to determine not only the actual conversion pathway, but the rate of conversion as well. Similarly, isotopic tracers have shed light on the origin of important excretion products, such as urea, creatine, urinary ammonia and the like.

With some of the radioactive isotopes, it is possible to follow the course of a marked compound in the intact body, thus obviating the necessity for sacrificing the animal and removing tissues for analysis. In such cases a sensitive measuring device such as the Geiger counter is placed over the region of the body where the accumulation of the tagged compound is to be investigated. By this means it is possible to record and follow the ebb and flow of the tagged compound during metabolism. This procedure is of particular value in human metabolism studies.

Another useful method for detecting the presence and measuring the concentration of radioactive tracers especially where fine detail is important, as in tissue analysis, involves the taking of radio-photographs of tissues in which the tracer substance has accumulated.

Metabolism of Fats:

Some of the earliest biological experiments with isotopic compounds were carried out ten years ago by Schoenheimer, Rittenberg and their associates (1) on fats.

In mammals most of the fat is deposited under the skin, between muscles or around internal organs. This fat is known as storage or depot fat and had previously been regarded as being essentially inert, constant in composition, and drawn into metabolic processes only under stress of emergencies such as starvation. Energy was thought to be derived directly from dietary fat and other food constituents, of which excess supplies were relegated to the inert deposits of storage fat. The work of Schoenheimer's group soon brought to light the amazing fact that *the so-called "inert" stores of fat are in a state of constant, rapid flux and that dietary fat and depot fat are in continuous dynamic equilibrium.*

The first series of their experiments on the metabolism of dietary fats was carried out with a fat prepared by hydrogenating linseed oil with deuterium, the stable heavy isotope of hydrogen (9). This tagged fat was fed at a level of 2% of the diet to normal adult mice receiving a low-fat but otherwise normal diet. At the end of four days the animals were sacrificed and the body fat analyzed for the presence of the deutero-fat. Almost half of the tagged dietary fat (44%) had found its way into the storage fat, yet the total fat reserves remained constant! This could only be interpreted as an indication of rapid fat replacement. The implication was obvious—an amount of depot

fat corresponding to that newly deposited must have been degraded and utilized. In other words, the fats which the animals burned were not merely absorbed dietary fats, but consisted of an almost equal mixture of ingested food fats and storage fats.

In order to follow more precisely the role of fat in metabolism it was next decided to trace the course of a single, pure fatty acid. Accordingly, in the next experiment (10) labeled palmitic acid containing 22 atom per cent deuterium (i.e. 22% of the hydrogen atoms were replaced by deuterium) was fed. To offset the possibility that some of the fat deposition in the first experiment with low-fat diets might have been due to the nutritional requirements of the animals for special fatty acids, an adequate amount of butter, selected because it contains all the essential fatty acids, was included in the diet of the next experiment. After eight days, 44 per cent of the body fats were replaced by the labeled fatty acid. Whether this represented a replacement of palmitic acid in the depot fat by a deposition of the labeled dietary fat as such or whether the deuterium had found its way into other types of fatty acids which, in turn, had replaced their analogues in the depot fat was the next question which arose. Analysis of the various fatty acids isolated from the depot fat soon proved the latter to be the case. Most of the isotope was concentrated in the palmitic acid fraction, but part of the palmitic acid had been degraded to form several shorter-chained fatty acids and part had condensed with another compound to synthesize longer-chained fatty acids as evidenced by the deuterium content of these newly-formed acids. No deuterium was found, however, in linoleic acid thus supporting the findings of Burr and Burr (11) that this is an indispensable food constituent which cannot be synthesized within the body.

These experiments offered conclusive evidence for the biological desaturation and conversion of one fatty acid into others. They also indicated that this interconversion of fatty acids occurs in the body even if the fatty acids formed are supplied plentifully in the diet as they were with the inclusion of butter.

Further experiments showed that fats can be synthesized in the body from other dietary materials and that this formation is a continuous and rapid process irrespective of the presence or absence of dietary supplies of fat (12, 13). This was done by feeding animals on a carbohydrate diet "heavy" water (D_2O instead of H_2O) which eventually became equally distributed in all the body fluids. All chemical reactions in the body thus occurred in a medium of heavy water. The fatty acids synthesized during this period all contained deuterium. The reversibility of this process was shown when other mice, previously fed a deuterio-fat, were placed on a similar carbo-

hydrate diet, but received ordinary water to drink. After the feeding of the labeled fat had been discontinued, the isotope level of the fatty acids fell at the rate almost identical with that found for the synthesis. The fact that the deuterium was introduced during fatty acid formation and not by physical exchange in pre-existing fatty acids was demonstrated by the presence in the same animal of deuterium in some fatty acids, but not in those which cannot be synthesized by the body (14).

The appearance of the isotope marker in fatty acids further provides a method not only to indicate which acids are formed but to show how rapidly the reaction occurs with different acids (13). *In vivo* synthesis of lipid compounds other than the fatty acids, such as cholesterol and other phospholipids has likewise been demonstrated by this method.

As a result of these studies it has been shown that all the lipids of the fat depots are constantly subject to a great variety of highly complex chemical processes—synthesis, interconversion and degradation. Schoenheimer (1) has termed the sum of the biological reactions in which an organic compound is involved without ultimate change in amount or constitution, "molecular regeneration." He concluded that:

> "The fatty acids of the depot fat are to be regarded as being constantly transported, in the form of fats or phosphatides, to and from the organs, where fatty acids are temporarily liberated by rupture of ester linkages. When fat is absorbed, the acids of dietary origin merge with those from the depot, thereby forming a mixture indistinguishable as to origin. Parts of the liberated acids are converted into others, while new ones are steadily formed by condensation of small molecules derived from other substances. Some of this pool of acids is degraded, and some of it re-enters ester linkages to regenerate fat, then transported back to the depots. All these complex reactions are so balanced that the total amount and structure of the fat mixture in depot, blood, and organs remain constant."

Proteins and Amino Acids:

The intermediary metabolism of proteins has been investigated with deuterium and N^{15}, the stable isotopes of hydrogen and nitrogen. Because of the highly specific character and colloidal size of the protein molecules it had been assumed, as in the early theories of fat metabolism, that they were stable chemical entities, invariant within the body. Elucidation of the metabolic behavior of proteins through the use of isotopic tracers here again has entirely revised earlier concepts of endogenous and exogenous nitrogen metabolism.

The proteins are among the most complex natural organic compounds and have molecular weights ranging from about 35,000 to

several millions. The huge protein molecule is built up of a number of amino acids held together in somewhat of a chain formation by peptide linkages (i.e. the joining of the amino group of one acid to the carbon chain of another amino acid through the linkage of the nitrogen and carbon atoms of each group, respectively). During the process of metabolism these peptide linkages are broken and the two parts of the amino acid may travel different pathways. In studying the metabolic course of a particular amino acid, therefore, it is necessary to use independent tracers for each part—N^{15} for the amino group and deuterium for the carbon chain.

Feeding amino acids thus doubly marked with N^{15} and deuterium, Schoenheimer *et al* (15, 16) were able to show that entire amino acids can be replaced as such in the tissue proteins and further, that dietary nitrogen supplied through labeled amino acids is readily transferred from some amino acids to certain others. These transfers and replacements of individual amino acids within a single protein molecule must necessarily involve the constant breaking open of existing peptide bonds and the creation of new linkages.

In a typical series of experiments, the pathway of natural amino acids was investigated by adding small amounts of their isotopic analogues to the stock diet of rats. In these experiments labeled leucine (15) and glycine (16) were added, singly, to diets containing ample amounts of protein and fed to animals which were in nitrogen equilibrium and maintaining constant weight. The excreta were collected and at the end of the test period of three days, the organs were analyzed to determine the isotope content of their nitrogen compounds. According to the concept of independent exogenous and endogenous metabolism, most of the dietary nitrogen should have appeared directly in the urine. This was not the case. Less than one-third of the isotopic nitrogen of the dietary leucine and less than one-half of that of the dietary glycine appeared in the excreta. The major fraction of the retained isotopic nitrogen appeared in the protein fraction of the tissues throughout the various parts of the body. Other experiments confirmed the fact that the results with leucine and glycine are typical for all amino acids (1, 17, 18). This means that the tissue proteins are involved in a very rapid chemical exchange with at least half of the dietary nitrogen. This exchange is so balanced as to avoid net change in the nature and total amount of body proteins.

Isotopic analyses showed that different organs vary in their ability to fix dietary nitrogen. The proteins of the internal organs, of serum and of the intestinal tract are most active as acceptors of dietary nitrogen; the proteins of the muscles, less active and those of the skin, least active.

The use of isotopic tracers has also shown that dietary amino acids are utilized in two ways. The major portion is transferred as intact amino acids to the tissue protein with replacement of identical amino acid molecules. The nitrogenous portion of the remainder is used in the synthesis of most of the other amino acids which are then also incorporated into the tissue proteins, the sole exception being the amino acid lysine. When tagged lysine is fed its nitrogen can be used for the synthesis of other amino acids, but the reverse process never occurs.

As in the case of fatty acids, the synthesis of the various amino acids is found to take place continuously even when they are already abundantly supplied in the diet.

These studies marked the beginning of a large number of experiments designed to clarify the intermediate metabolism of proteins and amino acids. The various steps in amino acid degradation—enzymatic dehydrogenation to the imino form, spontaneous hydrolysis of the imino form to the ccrresponding keto form plus ammonia, and the reverse procedures, as well as the conversion of the ammonia to urea, have been demonstrated. The role of ammonia as an intermediate in amino acid synthesis has been indicated as a strong possibility. The biological occurrence of transamination* also has been confirmed through the use of the isotope technique. Isotope experiments have shown, likewise, that highly specific proteins such as serum antibodies, as well as the ill-defined mixtures of tissue proteins, are in constant interaction with the dietary amino acids. This confirms the theory of Whipple and associates (21) of a dynamic equilibrium between the proteins of blood and those of the tissues.**

A further result of experimental investigations with isotopes has been the elucidation of the concept of nutritionally essential or indispensable amino acids. Rose (27), using growth as a criterion, has defined an indispensable amino acid as one which the animal either cannot synthesize at all or not rapidly enough for normal growth. Schoenheimer *et al* (22, 23, 24, 25, 26) have given a chemical interpretation of this picture. They found that in certain cases an essential amino acid, such as lysine, constitutes a complete molecular entity which must be supplied as such in the diet. Most of the other amino acids, however, are indispensable only with respect to their carbon chain structures, their nitrogenous portions being obtainable from other sources.

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*A reaction resulting in the transfer of nitrogen among certain amino acids in the body without ammonia as an intermediate (19, 20).

**cf. *Borden's Review of Nutrition Research*, May, 1943—"The Influence of Dietary Factors on the Regeneration of Blood Plasma Proteins."

Thus, as with the fats and fatty acids, the proteins and amino acids of all the body tissues are undergoing constant changes. These involve degradation and synthesis (deamination and amination), nitrogen transfer and the breaking open of peptide linkages with the insertion of new fragments. All of these changes involve an equilibrium exchange between dietary and tissue proteins, so that the rapid and continuous flux of molecular regeneration of the body constituents represents an almost equal merging of dietary and existing tissue components.

Carbohydrate Metabolism:

The intermediary metabolism of carbohydrates has not been as clearly established as that of proteins and fats. Full application of isotope techniques has been hampered by the unavailability of the carbon isotopes.

Several important findings have already been obtained, however. Thus Conant *et al* (28) have shown that labeled bicarbonate and labeled lactic acid can yield labeled liver glycogen. Also, Wood, Werkman and associates (29) and Evans and Slotin (30) showed that isotopic carbon dioxide reacts with pyruvic acid to give oxalacetic acid.

These findings are at variance with previous conceptions of intermediate carbohydrate metabolism. Complete elucidation of these complex problems awaits further experimental work.

New Aspects of Nutrition Research:

While the fundamental studies on the metabolism of fats, proteins and amino acids were going on in Schoenheimer's laboratory, investigators in other laboratories were exploring different avenues of research with the aid of the new investigative tool—the isotopic tracer.

Outstanding among these investigations were the experiments of du Vigneaud and his group at Cornell University Medical School (31) out of which arose a new concept in nutrition—the dietary essentiality of labile methyl groups and their significance in the newly recognized biological process of transmethylation.

The origin of the transmethylation theory with the discovery of the biological function of the labile methyl group constitutes one of the most fascinating stories of modern nutrition research and has been described in an earlier issue of this *Review**.

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*cf. *Borden's Review of Nutrition Research*, October, 1942 — "Labile Methyl Groups — a Newly Recognized Nutritional Essential."

Briefly, the researches of du Vigneaud began with investigations into the dietary indispensability of certain amino acids along the classic lines of study initiated by Rose. During the course of these studies, it was found that the essential amino acid, methionine, could be replaced in the animal diet by the nonessential, homocystine, *if choline were simultaneously present in the diet* (32). Since the only difference between methionine and homocystine is in the methyl group content of the former, it was concluded that choline (a simple non-protein compound containing three labile methyl groups) had donated the requisite methyl groups to homocystine, thereby converting it to methionine.

As these experiments progressed, further interrelations between methylated and non-methylated compounds were uncovered. Methionine, for example, was found to donate its methyl groups to ethanolamine to form choline. This led to the explanation of the effectiveness of methionine in preventing fatty livers and hemorrhagic kidneys resulting from choline deficiency. These interrelationships led to the conclusion by du Vigneaud that the animal organism is incapable of synthesizing methyl groups for essential biological methylations and, therefore, that methyl groups in a utilizable, labile form must be provided in the diet.

Final and unequivocal proof for the postulated occurrence of biological methyl transfer was obtained through the use of isotopic tracers. This was accomplished by the feeding of compounds in which the methyl groups (CH_3) were labeled with deuterium (CD_3). The subsequent isolation of other compounds in which the isotope concentration was remarkably high, clearly indicated that the source of the isotope was the dietary methyl.

The use of isotopic tracers provided the answer to still another question, namely the explanation for the lipotropic action* of choline, methionine and other methylated compounds. This was found to be due to the stimulating effect of these compounds on the rate of phospholipid formation in the liver (33, 34, 35), (as evidenced by the occurrence of the tracer in the newly formed phospholipids) a process whereby liver fat deposits are converted to a utilizable form and thereby prevented from accumulating.

Conclusion:

Isotopes have furnished for the first time a tool for the investigation of the nature and rate of chemical reactions going on in the tissues of living animals.

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*An action resulting in the prevention and cure of fatty livers.

In the case of organic compounds—fats, proteins and carbohydrates—which are composed chiefly of carbon, hydrogen, oxygen and nitrogen, most of the work to date has been with the stable isotopes of these elements. Except in the case of radioactive carbon, C^{14}, and radioactive hydrogen, H^3, no radioactive isotopes with sufficiently long lifetimes are available at present for such experiments. The radioactive isotopes of inorganic elements—phosphorus, calcium, iron, iodine, sodium, potassium and others—on the other hand, have been extremely valuable in elucidating the role of these elements and of certain of the vitamins in animal metabolism. The interesting discoveries resulting from their use will be discussed in the forthcoming issue of this *Review*.

The most significant principle to arise as a result of the investigation of intermediary metabolism with isotopic tracers is the concept of *dynamic equilibrium* between absorbed food constituents and tissue components. The large and complex molecules of the body tissues are being continuously degraded into specific component units. These merge with similar units formed by the degradation of dietary materials. From this combined pool is regenerated the complex structure of body tissues. Even the most seemingly inert tissues are involved in this continuous interchange which is balanced so delicately that the body components remain constant in total amount and in structure.

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